PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Kenya SHITARA *et al.*

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For: DIAGNOSTIC AGENT AND THERAPEUTIC AGENT FOR LEUKEMIA

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir/Madam:

I, Dr. Kenya SHITARA, a citizen of Japan, do hereby declare as follow:

I graduated from the University of Tokyo, Faculty of Pharmaceutical Science in 1982, entered the graduate school of the University of Tokyo immediately after graduated, and got MSc degree in 1984. My major subject in the University of Tokyo was immunology. Since 1984, I have worked at Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd. At Tokyo Research Laboratories, I have been studying on establishment and evaluation of anti-tumor monoclonal antibodies. I got Ph.D. degree from the University of Tokyo, Faculty of Pharmaceutical Science in 1990. During 1993 and 1994, I stayed Neurobiology Program, La Jolla Cancer Research Foundation (Present name is The Burnham Institute) U.S.A. and studied on function of the novel proteoglycan in the brain in the lab. Since 1997, I have been a senior researcher and been at the position of the head, Division of Immunology, Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd. I belong to the Japanese Association for Cancer Research, and the Japanese Association for Metastasis Research. The number of my oral and poster presentations in the academic meetings of these and other international societies, such as the International Conference on AACR-NCI-EORTC and International Symposium on Cancer Chemotherapy are more

than twenty in total. My publication in academic journals are more than thirty; these journals include Journal of Biological Chemistry, Cancer Research, Journal of Immunology, Blood, Oncogene, Proceedings of National Academy of Science USA, etc.

I am familiar with the prosecution history of the above-identified patent application.

The following experimentation was conducted by me or my direct supervision.

ADCC activity:

(1) Preparation of target cells

As target cells, T-cell leukemia cell line HSB-2 (registered as CCRF-HSB-2 at ATCC, ATCC CCL-120.1) or T-cell leukemia cell line Jurkat (RCB0806) was cultured by a conventional method, and cells which reached the objective number at the logarithmic growth phase were recovered, followed by washing with RPMI1640 medium containing 10% fetal calf serum (hereinafter referred to as "10S-RPMI") twice. After the washing, the cell number was measured and suspended in 10S-RPMI at a concentration of 2×10^6 cells/ml. To the cell suspension, 3.7 MBq of sodium chromate (^{51}Cr) was added, and the target cells were isotope-labeled by keeping them at 37°C for 1 hour. After the labeling, the cells were washed with 10S-RPMI and suspended in 10S-RPMI at a concentration of 2×10^5 cells/ml to obtain a target cell suspension.

(2) Preparation of effector cells

From a healthy person, venous blood was collected in the presence of heparin sodium, and a mononuclear cell layer was isolated by using Lymphoprep (manufactured by Nycomed) in accordance with the manufacture's instructions. After washing with 10S-RPMI twice, suspension was carried out at a concentration of 5×10^6

cells/ml, 1×10^7 cells/ml or 2×10^7 cells/ml by using 10S-RPMI to obtain an effector cell suspension.

(3) Preparation of antibody solution

An anti-Flt-1 human chimeric antibody KM2550 (WO99/60025) was diluted with 10S-RPMI to an appropriately diluted antibody solution. As the negative control, anti-interleukin-5 receptor α-chain human chimeric antibody KM1399 (WO97/10354, corresponding to U.S. Patent 6,018,032) was used.

(4) Measurement of ADCC activity-1

Into each well of a 96 well U-shaped bottom plate (manufactured by Falcon), 50 μl of the target cell suspension of CCRF-HSB-2 or Jurkat prepared in the above (1) (1×10^4 cells/well) was dispensed. Next, 100 μl of the effector cell suspension prepared in the above (2) was added thereto (5×10^5 cells/well, 1×10^6 cells/well, or 2×10^6 cells/well, the ratio of the effector cells to the target cells became 200:1). Subsequently, the antibody solution was added thereto to give a final concentration of 50 μg/ml, and the total volume was made up to 200 μl, followed by reaction at 37°C for 4 hours. After the reaction, the plate was centrifuged, 75 μl of the supernatant was collected, and γ ray radioactivity of each supernatant was measured with a γ-counter.

The data of the spontaneously released target cells were obtained in the same manner as the above, except that the medium alone was used instead of the effector cell suspension and the antibody solution. The data of the total released target cells were obtained in the same manner as the above, by using 100 μl of 1N hydrochloric acid instead of the antibody solution and the effector cell suspension and

making the total volume up to 200 µl using 10S-RPMI The ADCC activity was measured by the following equation.

$$\text{ADCC activity (\%)} = \frac{\left(\begin{array}{c}\text{Data of antibody-}\\\text{added group}\end{array}\right) - \left(\begin{array}{c}\text{Data of}\\\text{spontaneously}\\\text{released target cells}\end{array}\right)}{\left(\begin{array}{c}\text{Data of total}\\\text{released target cells}\end{array}\right) - \left(\begin{array}{c}\text{Data of}\\\text{spontaneously}\\\text{released target cells}\end{array}\right)} \times 100$$

The results are shown in Fig. 1. The ordinate and the abscissa in Fig. 1 show the ADCC activity and the ratio of the effector cells to the target cells (ET ratio), respectively. As shown in Fig. 1, it was confirmed that the anti-Flt-1 human chimeric antibody KM2550 has ADCC activity against the two kinds of the leukemia cell lines (Jurkat and HSB-2) which express Flt-1, depending on the ET ratio.

(5) Measurement of ADCC activity-2

The activity of the antibody depending on the concentration was examined by using, as the target cell, Jurkat in which the ADCC activity of the anti-Flt-1 human chimeric antibody KM2550 is higher in comparison with CCRF-HSB-2.

Into each well of a 96 well U-shaped bottom plate (manufactured by Falcon), 50 µl of the target cell suspension of CCRF-HSB-2 or Jurkat prepared in the above (1) (1×10^4 cells/well) was dispensed. Next, 100 µl of the effector cell suspension prepared in the above (2) was added thereto (1×10^6 cells/well, the ratio of the effector cells to the target cells became 100:1). Subsequently, the antibody solution was added thereto to give a final concentration of 0.5 µg/ml, 5 µg/ml or 50 µg/ml and the total volume was made up to 200 µl, followed by reaction at 37°C for 4

hours. After the reaction, the plate was centrifuged, 75 μl of the supernatant was collected, and γ ray radioactivity of each supernatant was measured with a γ-counter.

The data of the spontaneously released target cells and the data of the total released target cells were obtained in the same manner as in the above (4). The ADCC activity was measured by the above equation.

The results are shown in Fig. 2. The ordinate and the abscissa in Fig. 2 show the ADCC activity and the antibody concentration, respectively. The white bars and the black bars show the ADCC activities of the anti-Flt-1 human chimeric antibody KM2550 and the anti-interleukin-5 receptor α-chain human chimeric antibody KM1399 as the negative control, respectively.

The results show the antibody KM2550 has the ADCC activity depending on the antibody concentration. Whereas, it was shown that the antibody KM1399 as the negative control does not have the ADCC activity depending on the antibody concentration.

Based on the above results, it was shown that the anti-Flt-1 human chimeric antibody KM2550 has ADCC activity against leukemia cell lines.

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I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: June 3, 2004 Name: Kenya Shitara

Kenya SHITARA